IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Kwoh et al.	
Serial No.:	09/523,033	ART UNIT: 1645
Filed:	March 10, 2000	
Entitled:	METHOD FOR INCREASING HDL CHOLESTEROL LEVEL	EXAMINER: A. Navarro

Atty. Docket No.: TCS-428.0 US-1

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

DECLARATION OF LAWRENCE J. THOMAS, Ph.D.
UNDER 37 C.F.R. §1.132

I, Lawrence J. Thomas, hereby declare and state that:

1. I am the Associate Director, Pharmacology/Toxicology, at AVANT IMMUNOTHERAPEUTICS, INC., Assignee of the above-referenced patent application, U.S. application Serial No. 09/523,033.

2. I am familiar with the disclosure of U.S. application Ser. No. 09/523,033. In addition, I have either conducted or supervised scientific experiments related to the development and administration of immunogenic vaccine compositions comprised of a full-length cholesteryl ester transfer protein (CETP) linked to a carrier, which vaccine compositions, when administered to a mammalian subject, unexpectedly elicited the production of antibodies that, in addition to recognizing the vaccine immunogen, also recognized and bound to the vaccinated subject's endogenous CETP, leading to effective modulation of CETP activity.

3. I have read the Office Action issued May 27, 2005 in the subject Kwoh et al. application Ser. No. 09/523,033. I have also read and understand the subject matter and claims currently pending in the subject Kwoh et al. application.

4. I have been informed by AVANT's attorneys, and it is my understanding, that Claims 1, 2, 5, 9, 11, and 13 of the Kwoh et al. application have been rejected as being anticipated by Swenson et al., Mechanism of Cholesteryl Ester Transfer Protein Inhibition by a Neutralizing Monoclonal Antibody and Mapping of the Monoclonal Antibody Epitope, *The Journal of Biological Chemistry*, 264 (24): 14318-14326 (1989) ("Swenson"). The Examiner has also rejected Claims 1, 2, 5-7, and 9-13 as being obvious in view of the combined teachings of Swenson (above), further in view of Nagashima et al., Cloning and mRNA Tissue Distribution of Rabbit Cholesteryl Ester Transfer Protein, *Journal of Lipid Research*, 29: 1643-1649 (1988) ("Nagashima") and Maciak et al., U.S. Pat. No. 5,264,341, Selective Cloning For High Monoclonal Antibody Secreting Hybridomas ("Maciak"). I have been informed and believe that an important issue in the Office Action relates to whether these cited references are sufficient to suggest or make obvious the methods described in the Kwoh et al. application for administering a vaccine peptide composition comprised of a full-length CETP protein conjugated to a carrier peptide, which composition, when administered to a mammalian subject elicits the production of autoantibodies against that subject's endogenous CETP.

5. I believe that I have performed experiments and am in possession of scientific data related to the surprising and unexpected ability of CETP vaccines comprised of a full-length CETP protein conjugated to a carrier to elicit an auto-immune response in the vaccinated subject effective to modulate the endogenous CETP activity of the subject, which data may be relevant to the inventiveness of the claims in the above-referenced patent application. No similar data are present in the cited publications of the Office Action, and thus there is no published data to advise practitioners in this field that such an auto-immune response is likely to

occur with the use of the immunogenic vaccine composition discussed in the Kwoh et al. application.

6. The Kwoh et al. application claims methods for increasing HDL-cholesterol (HDLc) levels by actively immunizing a subject with a vaccine composition comprising a full-length CETP protein conjugated to a carrier. My data show that the use of such a vaccine has a remarkable effect on the lipoprotein profile of the vaccinated subject. Specifically, my data show that administration of a vaccine composition according to the invention to a mammalian subject, as taught by Kwoh et al., results in the generation of autoantibodies against the subject's endogenous CETP. In addition to the generation of autoantibodies, my data demonstrate the surprising discovery that generation of these autoantibodies against endogenous CETP results in an overall decrease in the percent of total cholesterol present in the bloodstream, an overall decrease in LDL-cholesterol (so-called "bad" cholesterol), and, even more surprisingly, a significant reduction in the area of atherosclerotic lesions present in the arteries of subjects fed a high cholesterol diet following administration of the vaccine composition, as compared with a control subjects not receiving the vaccine composition.

7. I have personally conducted or supervised experiments where I conjugated a full-length human CETP protein to a carrier peptide, e.g., a portion of the tetanus toxin, as an immunogenic carrier. As described below, I vaccinated New Zealand White rabbits with a vaccine composition comprising either: a full-length human CETP conjugated with a tetanus toxoid peptide (conjugate), or human chorionic gonadotropin (irrelevant antigen) as a negative control. After vaccination, serum was collected from each rabbit and tested for the presence of antibodies generated by the vaccination and their ability to bind endogenous rabbit CETP, overall changes in cholesterol levels, changes in the level of LDL-cholesterol in the serum, and finally, an analysis of the overall area of atherosclerotic plaque development in the arteries of vaccinated rabbits fed a high cholesterol diet after administration of the vaccine.

8. It is well known in the art that human and rabbit CETP share an 80% amino acid homology and 75% of the non-homologous amino acid sequences are conservative substitutions. Therefore, the vaccine composition of this experiment represented vaccination with a CETP-carrier immunogen wherein the CETP structures were very similar to the native CETP of the subject.

9. **I. Immunization of Rabbits With Full-Length Human CETP Conjugated to a Full-Length Carrier Protein**

A. Immunization of Rabbits Against Endogenous CETP

Two vaccine preparations were made for injection into two groups of twelve New Zealand White Rabbits, to test the ability of the vaccine preparation to elicit an immune response against endogenous CETP. Group I (negative control) included rabbit #'s 1-12, each of which was injected with a vaccine composition containing an irrelevant immunogen, human chorionic gonadotropin (HCG). Group II included rabbit #'s 13-24, each of which received a vaccine composition comprising a whole recombinant human CETP protein conjugated with tetanus toxoid via a chemical crosslinker ("Conjugate").

B. Vaccine Administration

The vaccination protocol was as follows: On Day 1, each rabbit received one subcutaneous injection of a composition containing 200μg of immunogen in Complete Freund's Adjuvant (Sigma Chemical Co., St. Louis, Missouri). Each composition was suspended in phosphate buffered saline (PBS) and emulsified with Complete Freund's Adjuvant (1:1) to yield a final concentration of 100μg/100 μl. Each rabbit was administered the vaccine mixture in a 200μl dose (200μg immunogen) at one subcutaneous site. Boosts of 200μg of immunogen in Incomplete Freund's Adjuvant (Sigma Chemical Co.) were administered subcutaneously at Weeks 5 and 8 after the initial vaccination. At Week 16, a further boost was administered and all rabbits were switched to a 0.25% cholesterol diet. Blood samples (approximately 1-5ml) were withdrawn from the

ear of each rabbit prior to each initial injection ("prebleed") and at Weeks 5, 8, 10, 12, 14, 16, 19, 22, and 27. The animals were sacrificed at Week 32. Blood plasma samples were prepared by standard centrifugation methods to separate cellular components from the plasma. Plasma samples were stored frozen at -70°C. Plasma samples of both Groups I and II were analyzed for the presence of and increase in titer of anti-CETP antibodies, plasma levels of various lipoprotein components (total cholesterol and LDL-cholesterol), and the effects of the vaccine on the development of atherosclerotic lesions in the arteries of rabbits fed a high cholesterol diet following administration of the vaccine composition as compared with the arteries of control rabbits. The results of these experiments are shown in Exhibits A-E (Tabs A-E), attached to this declaration.

C. Direct ELISA for Titering Anti-CETP Antibodies

A sandwich enzyme-linked immunosorbent assay (ELISA) was used to titer plasma samples containing anti-CETP antibody. A biotinylated C-terminal peptide (20 amino acids) of rabbit CETP was adsorbed to wells of a microtiter dish coated with streptavidin. Various dilutions of rabbit plasma collected from the rabbits of Groups I and II were then added to each well. Non-specific binding was blocked by addition of a 1% solution of BSA in PBS and 0.05% Tween to each well, followed by incubation for 2 hours at room temperature (20-22°C) on a rotating shaker at 150 rpm. The wells were then washed four times with ELISA wash buffer (PBS + 0.05% Tween). Plasma samples were then diluted 1:10 in dilution buffer (1% BSA in PBS), followed by serial dilutions in the same buffer. Diluted samples (100µl) were added to the wells, incubated for 2 hours at room temperature on a rotating shaker at 150 rpm, and then washed 4 times with ELISA wash buffer (PBS + 0.05% Tween). To detect bound anti-CETP antibodies, 100 µl of a 1:10,000 dilution of horseradish peroxidase (HRP) labeled goat anti-rabbit immunoglobulin (Southern Biotechnology Associates, Inc., Birmingham, Alabama) in dilution buffer was added and the plates incubated for 2 hours at room temperature on a rotating shaker at 150 rpm. The wells were then washed four times with ELISA wash buffer (see above), peroxidase substrate TMB (TMB

peroxidase substrate; from Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland) was added and the plates were incubated 30 minutes at room temperature. The change in optical density was monitored spectrophotometrically at 450 nm using an ELISA reader (e.g., E-max, Molecular Device Corp., Menlo Park, California). In this assay, the O.D. was directly proportional to the amount of rabbit anti-CETP antibodies present in the plasma samples. The results of the assay for each of the rabbit groups is shown in Exhibit A (Tab A). As seen in Exhibit A, Group I (control) showed no plasma antibodies detecting the rabbit C-terminal CETP peptide, whereas the Group II rabbits vaccinated with CETP immunogen (Conjugate) showed significant titers of anti-CETP antibodies present in the serum of almost all vaccinated rabbits.

Conclusion

The results in Exhibit A demonstrate that administration of a vaccine composition (comprising a full-length CETP conjugated to an immunogenic carrier) to a mammalian subject, elicited the production of autoantibodies that recognize and bind the vaccinated subject's endogenous CETP.

D. Cholesterol and HDLc Levels in Plasma Samples of Vaccinated Rabbits

The plasma samples taken from the rabbits of Groups I and II were assayed for the concentration of total cholesterol (Exhibit B), and the concentration of LDLc (Exhibit C) using standard commercial assays (Wako Chemicals USA, Inc., Richmond, Virginia). LDLc is calculated as total cholesterol minus HDL-cholesterol (HDLc) minus 0.2 × tryglyceride level. As seen in Exhibits B and C, the production of autoantibodies against endogenous CETP resulted in a dramatic decrease in the percent of total cholesterol and the percent of LDLc ("bad" cholesterol) present in the serum of the vaccinated subject as compared with control rabbits administered an irrelevant immunogen, HCG.

Conclusion

The data in Exhibit B (% change in total cholesterol) and Exhibit C (% change in LDLc), demonstrates a direct correlation between the production of autoantibodies against endogenous CETP (Exhibit A) and an overall reduction in

total cholesterol and LDLc in the serum of rabbits vaccinated with a composition in accordance with the Kwoh et al. invention.

E. Measurement of cholesterol deposits in the irises of vaccinated rabbits

The rabbits of Groups I and II were assayed for the amount of cholesterol deposits detected in the irises. A scale of cholesterol deposition in the iris was established, with 0 = no deposit, 1 = 20%, 2 = 40%, 3 = 60%, 4 = 80%, and 5 = 100% deposits on the iris. One iris per rabbit was evaluated and scored for degree of cholesterol deposition. Exhibit D shows the data collected from all the animals. The Xs are for animals for which no data was derived (rabbit #15 and #18). Exhibit D shows the score for each rabbit tested (vertical bars) and the average score for all the rabbits in Group I and all the rabbits in Group II (horizontal bars). As seen in Exhibit D, the CETP/carrier conjugate-vaccinated group had a statistically lower (by almost 30%) average of cholesterol deposits than the control group (40% vs. 70%, respectively).

Conclusion

The data in Exhibit D demonstrates a correlation between the production of autoantibodies against the endogenous CETP of a subject vaccinated with a composition according to the method of the subject Kwoh et al. application and the overall reduction in total cholesterol present in the vaccinated subject.

F. Quantitation of lesions in aortas of vaccinated rabbits

The rabbits of both groups were switched from a diet of basic rabbit chow to diets supplemented with various amounts of cholesterol, a diet known to produce atherosclerotic-like lesions in rabbits (Daley et al., *Arterioscler. Thromb., 14*: 95 - 104 (1994)). To determine whether the vaccination may affect the development of atherosclerosis, the aortas of these rabbits were examined histologically for the development of atherosclerotic lesions. After blood samples were taken on the last day, rabbits were sacrificed. The entire aortas from each rabbit of Groups I and II were removed and placed into fixative solution (3.7% v/v formaldehyde). Loose tissue, adherent fat, and the adventitia were dissected free from the arteries.

Each artery was then cut lengthwise, pinned flat to expose the intimal (luminal) surface, stained with Sudan IV, and then photographed. Sudan IV is a fat soluble red dye that stains atherosclerotic plaques on the intimal surface of arteries. The stained aortas of rabbits vaccinated with human chorionic gonadotropin ("HCG") revealed a prevalence of atherosclerotic lesions along the length of the aortas and particularly in the portion of the aortas from the thoracic region (data not shown). In contrast, the aortas of rabbits vaccinated with a full-length CETP/tetanus toxoid conjugate composition ("Conjugate") had a much smoother and more uniform appearance on the intimal surface owing to a lower incidence of lesions, including the portion of the aorta from the thoracic region (data not shown).

To quantify the noticeable difference in the presence of atherosclerotic lesions in the aortas of rabbits or lack thereof, the surface area of the pinned aortas and that of the aortic lesions was determined from photographs by planar morphometry (Daley et al., 1994) using a digitizing tablet with associated software (THE MORPHOMETER™, Woods Hole Educational Associates, Woods Hole, Massachusetts). The percentage of the surface area of the aortas covered by lesions was determined and the percentages are represented in Exhibit E. Open symbols represent the percent of the surface area covered by lesions in individual rabbits and the "+" symbol represents the median percent of arterial surface covered by lesions for each group as a whole. As seen in Exhibit E, Group I control rabbits had lesions present on approximately 25% more of the surface area of their arteries than the Group II rabbits administered the CETP/carrier vaccine composition (Conjugate).

Conclusion

Therefore, the results shown in Exhibit E demonstrate a further correlation between the generation of autoantibodies against endogenous CETP in subjects administered a vaccine composition comprising a full-length CETP protein conjugated to a carrier and a significant reduction in the development of atherosclerotic plaques present in the arteries of vaccinated subjects as compared with control subjects not receiving this composition.

10. Conclusions

The results shown above are remarkable in that by following the method of the claims of the Kwoh et al. application it was possible to induce an immune response against the vaccinated subject's endogenous CETP, resulting in a decrease in the amount of cholesterol circulating in the bloodstream, a decrease in the level of LDLc circulating in the bloodstream, a concommitant rise in the level of circulating HDLc, and a direct inhibition of the development of atherosclerotic plaques in the arteries of treated rabbits. The references cited by the Examiner in the Office Action of May 27, 2005 do not teach this vaccination protocol or predict the remarkable results described above.

11. I further declare that all statements made herein of my own knowledge are true and that statements made on information and belief are believed to be true and further that false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

11/22/2005
date

Lawrence J. Thomas, Ph.D.
AVANT IMMUNOTHERAPEUTICS, INC.

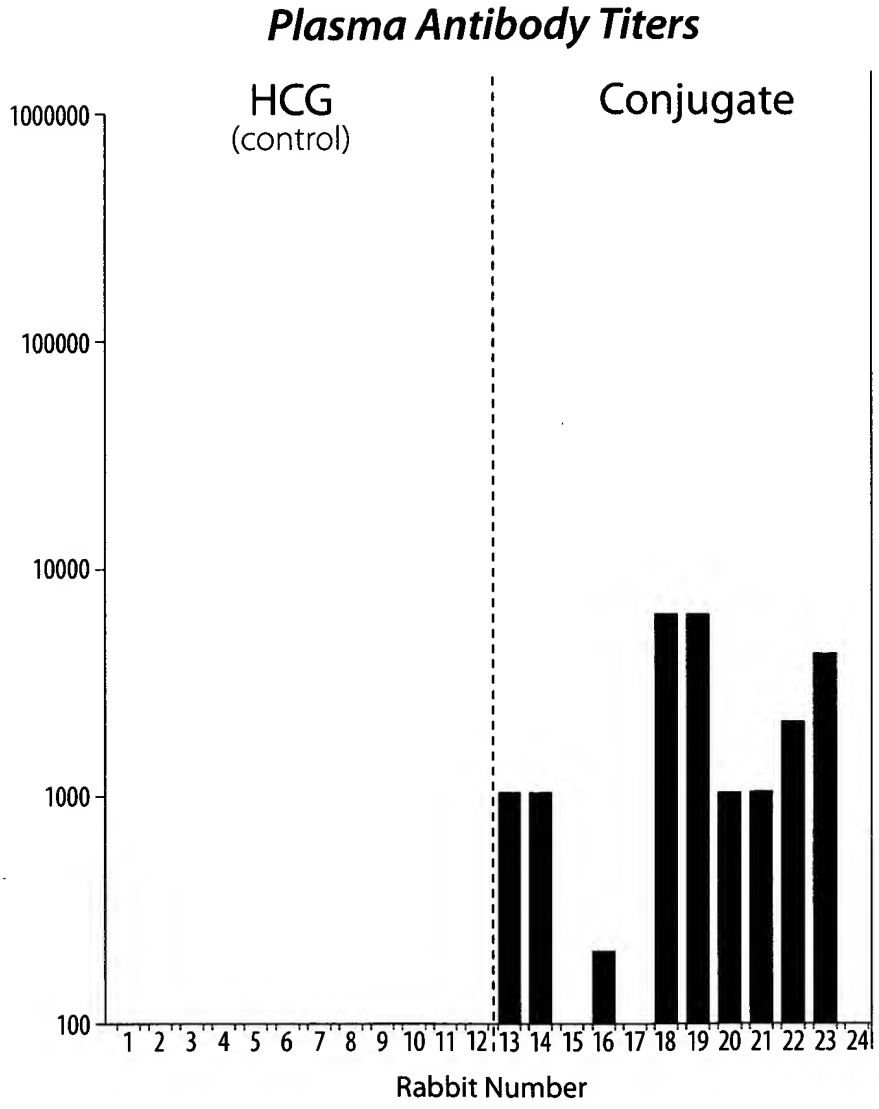
Plasma Antibody Titers
HCG
(control)
Conjugate
1000000
100000
10000
1000
100
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Rabbit Number

Exhibit A

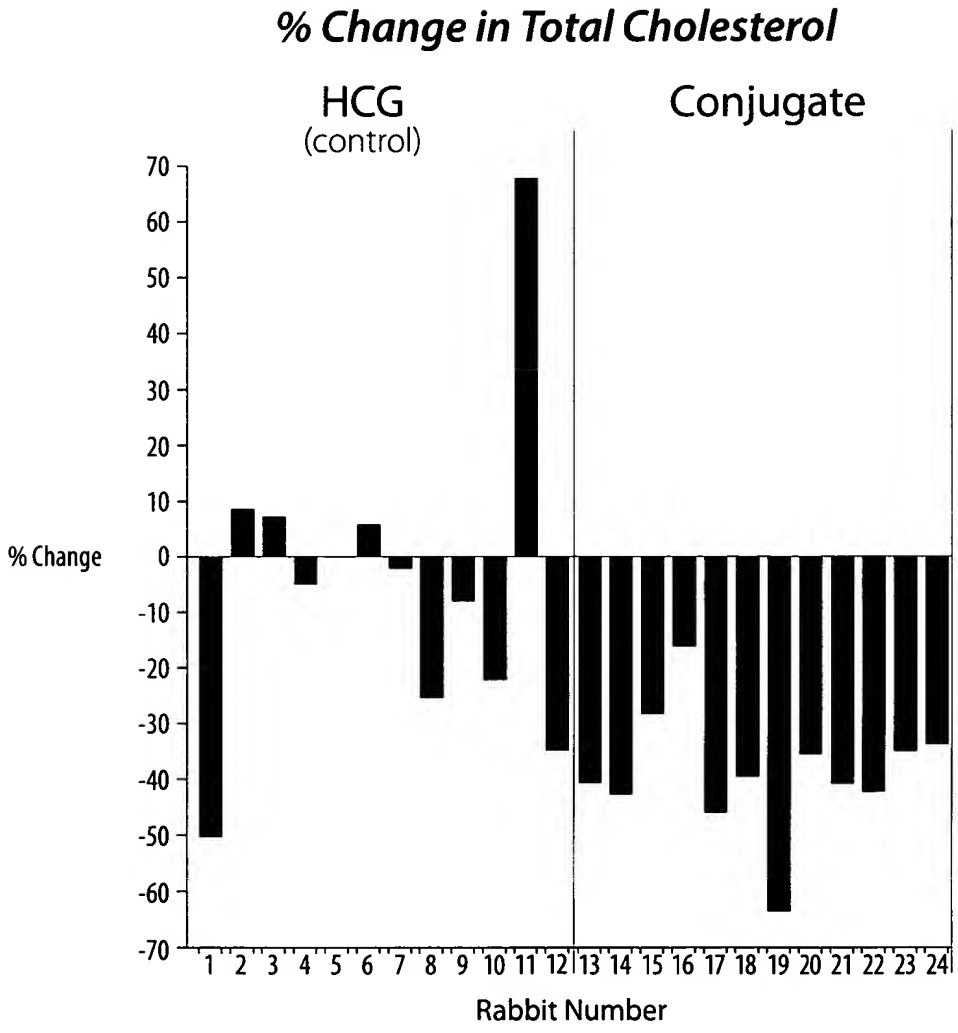
% Change in Total Cholesterol
HCG
(control)
Conjugate
% Change
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Rabbit Number

Exhibit B

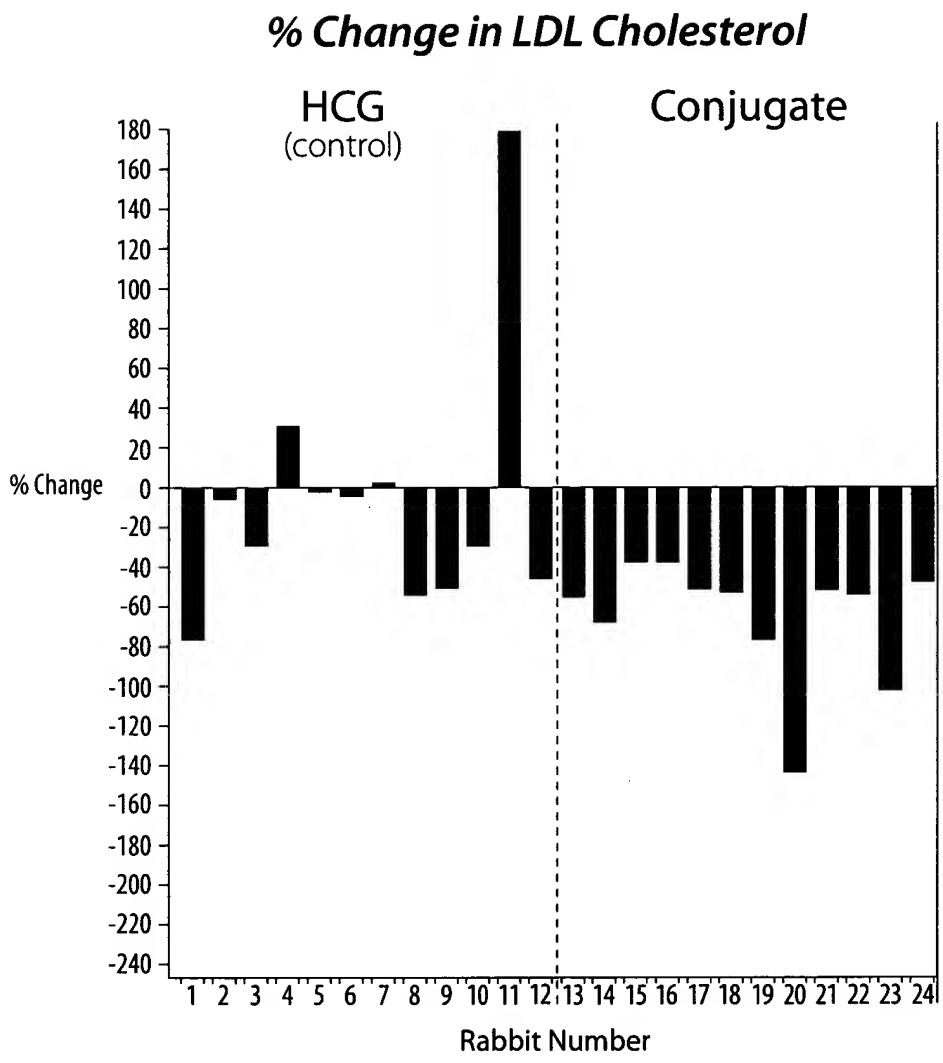
% Change in LDL Cholesterol
HCG
(control)
Conjugate
180
160
140
120
100
80
60
40
20
0
-20
-40
-60
-80
-100
-120
-140
-160
-180
-200
-220
-240
% Change
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Rabbit Number

Exhibit C

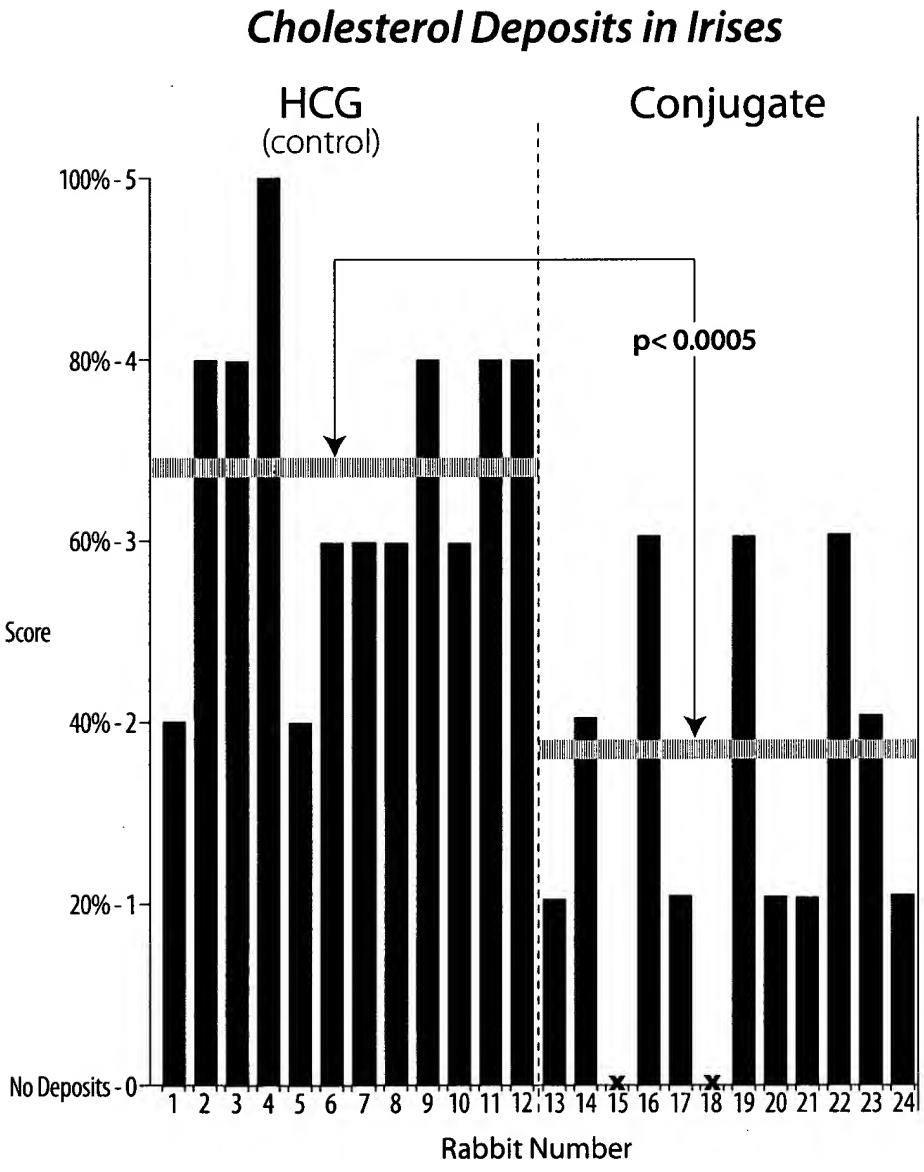
Cholesterol Deposits in Irises
HCG
(control)
Conjugate
100% - 5
80% - 4
60% - 3
40% - 2
20% - 1
No Deposits - 0
Score
p< 0.0005
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Rabbit Number

Exhibit D

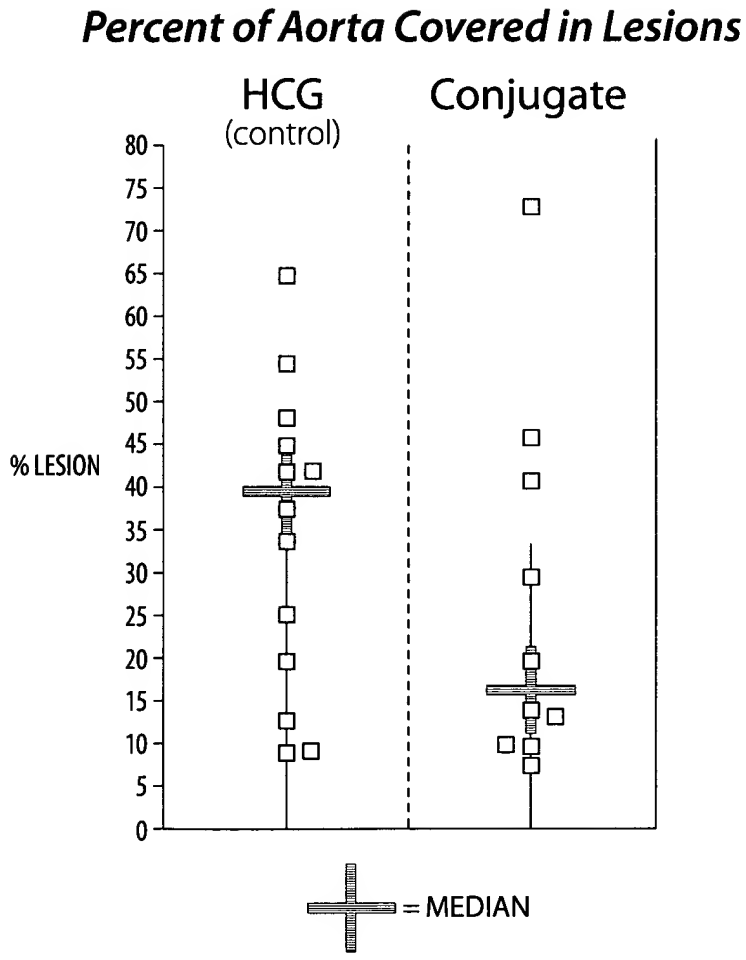

Exhibit E